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Reduction and S-Alkylation with Acrylonitrile

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Principle

This method is based on the fact that protein thiol groups, formed by reduction of disulfide bonds with mercaptoethanol, are effectively blocked by alkylation with acrylonitrile. The reaction is quantitative and the resulting cyanoethyl derivative is formed according to the following equation:

$$\underset{\text{Cysteine}}{HOOC—CH(NH_2)—CH_2—SH} + \underset{\text{Acrylonitrile}}{CH_2=CH—CN} \rightarrow \underset{S\text{-Cyanoethylcysteine}}{HOOC—CH(NH_2)—CH_2—S—CH_2—CH_2—CN} \quad (1)$$

Procedure

Protein (1 g) is dissolved in 20 ml water or urea solution adjusted to pH 8.0. To this solution is added with stirring a 40-fold molar excess of 2-mercaptoethanol (based on the total content of half-cystine per mole protein), and the pH is readjusted to 8.0 with dilute NaOH. The reaction mixture is left overnight at room temperature and under nitrogen. Twice the quantity of redistilled acrylonitrile, calculated relative to the amount of 2-mercaptoethanol originally used, is added. The pH is maintained at 8.0. After 4 hours the pH of the reaction mixture is adjusted to the isoelectric point of the protein, and the precipitated protein is dialyzed against distilled water. After dialysis the *S*-cyanoethylated protein is recovered quantitatively by lyophilization.

Acid Hydrolysis and Amino Acid Analysis

Samples of the *S*-cyanoethylated protein for acid hydrolysis are normally transferred to 15 × 125 mm Pyrex[2] test tubes. Constant-boiling HCl is added until the concentration of protein is approximately 2 mg per milliliter. The samples are frozen solid in a Dry Ice-acetone bath, after which the tubes are sealed under vacuum. Hydrolysis is carried out at 110° for 24 hours. Amino acid analyses of *S*-cyanoethylated protein hydrolyzates are performed by the manual chromatographic method of Moore, Spackman, and Stein,[3] or the automatic method of Piez and

[1] Deceased, December 7, 1964.

[2] Mention of specific firms and products does not imply endorsement by U.S. Department of Agriculture to the possible exclusion of others not mentioned.

[3] S. Moore, D. H. Spackman, and W. H. Stein, *Anal. Chem.* **30**, 1185 (1958).

Morris.[4] *S*-Cyanoethylcysteine is converted to *S*-carboxyethylcysteine during acid hydrolysis according to the following equation:

$$\underset{\text{S-Cyanoethylcysteine}}{\text{HOOC—CH(NH}_2\text{)—CH}_2\text{—S—CH}_2\text{—CH}_2\text{—CN}} \xrightarrow[\text{H}_2\text{O}]{\text{HCl}} \underset{\text{S-Carboxyethylcysteine}}{\text{HOOC—CH(NH}_2\text{)—CH}_2\text{—S—CH}_2\text{—CH}_2\text{—COOH}} + \text{NH}_4\text{Cl} \quad (2)$$

S-Carboxyethylcysteine has an elution volume of approximately 168 ml with the manual chromatographic procedure of Moore *et al.*,[3] while the volume is ~158 ml in the system of Piez and Morris,[4] as reported by Kalan *et al.*[5] The color yield for *S*-carboxyethylcysteine is 0.73 relative to leucine and the derivative gives a constant of ~17 when calculated by the height × width method of integration. Both chromatographic methods achieve separation and allow direct quantitation of *S*-carboxyethylcysteine.

Discussion

Alkylation of reduced proteins with acrylonitrile affords quantitative reaction with thiols.[6–8] However, it has been shown that, under certain conditions of pH and length of exposure of reduced protein to acrylonitrile, modification of lysine residues also occurs. Kalan *et al.*[5] and Woychik and Kalan[8] reported modification of ~25% of the lysine residues in β-lactoglobulin after 4 hours at pH 8.0, and a 45% loss in lysine after 15 minutes at pH 8.0 with κ-casein. It has been reported that an exposure time of 4 hours resulted in modification of 18% of the lysine residues of glutenin and 24% of those of gliadin.[9] Conversely, Plummer and Hirs[7] observed with ribonuclease that an exposure time of 2–4 hours at pH 8 had no effect on lysine residues, while at pH 9.5 modification of lysine residues occurred. It is likely therefore that the extent of involvement of lysine depends upon the particular steric factors associated with ε-amino groups in the protein.

Friedman and Wall[10] recently demonstrated that the alkylation of amino groups of model compounds with acrylonitrile is a base-catalyzed, nucleophilic addition, the rate of which is determined by the polar and steric characteristics of both reactants. They also found that the relative

[4] K. A. Piez and L. Morris, *Anal. Biochem.* **1**, 187 (1960).
[5] E. B. Kalan, A. Neistadt, and L. Weil, *Federation Proc.* **24**, 225 (1965).
[6] L. Weil and T. S. Seibles, *Arch. Biochem. Biophys.* **95**, 470 (1961).
[7] T. H. Plummer, Jr. and C. H. W. Hirs, *J. Biol. Chem.* **239**, 2530 (1964).
[8] J. H. Woychik and E. B. Kalan, *J. Dairy Sci.* **48**, 1113 (1965).
[9] M. Friedman, personal communication.
[10] M. Friedman and J. S. Wall, *J. Am. Chem. Soc.* **86**, 3735 (1964).

reactivities of SH and NH_2 groups with acrylonitrile, at comparable basicities and in equal steric environments, were 280:1 in model compounds. However, changes in pK and in steric environment cause this rate to vary widely. The variation found in the extent of alkylation of lysine residues in the proteins mentioned above would corroborate these findings.

The concentration of acrylonitrile may also be important in minimizing undesirable side reactions. In the procedure given above, a 2-fold excess of acrylonitrile over the free sulfhydryl groups in the reaction mixture represents approximately a 40-fold excess of acrylonitrile over the SH groups of the protein after all the mercaptoethanol has become alkylated. Therefore it is suggested that side reactions may be minimized by decreasing the concentration of acrylonitrile.

Plummer and Hirs[7] found that *S*-cyanoethylation was essentially complete in less than 20 minutes, in agreement with our own unpublished results. We have found that *S*-cyanoethylation is practically complete in less than 15 minutes. Therefore, if cyanoethylation of lysine residues is observed during a 4 hour period at pH 8.0, it is probable that if the time be reduced this side reaction will be minimized.

Several workers[7,11] have expressed inability to achieve resolution or separation of *S*-carboxyethylcysteine with analyzers operated according to Spackman *et al.*[12] Plummer and Hirs[7] have reported that *S*-carboxyethylcysteine sulfone, prepared by performic acid treatment of the sample prior to hydrolysis, may be separated by a procedure similar to that of Spackman,[13] and that the reaction is quantitative when carried out under the conditions used for determination of cystine as cysteic acid.[14]

[11] R. M. Zacharius and E. A. Talley, *Anal. Chem.* **34**, 1551 (1962).
[12] D. H. Spackman, W. H. Stein, and S. Moore, *Anal. Chem.* **30**, 1190 (1958).
[13] D. H. Spackman, *Federation Proc.* **22**, 244 (1963).
[14] S. Moore, *J. Biol. Chem.* **238**, 235 (1963).